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Advantages of hyaluronic acid as a component of fibrin sheet for care of acute wound

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ABSTRACT

Skin injury is followed by accumulation of a fibrin based provisional matrix which normally drives the process of wound repair. Exogenous fibrin with extra cellular matrix (ECM) components can also favor the wound healing process. In a preliminary study we found that lyophilized fibrin sheet (FS) arrest bleeding from rabbit skin wound but it remained dry during the repair period and did not accelerate the healing process better than untreated control. In the current study, hyaluronic acid (HA) was incorporated into FS and the resultant HA-FS promoted water retention and improved wound healing process. Gross-morphology, histopathology and histomorphometry were employed to compare qualitative and quantitative difference of wound healing in treated group against controls. In experimental sites (HA-FS), re-epithelialization was completed by 14 days with neo-vascularization and deposition of wavy bundles of collagen in the treated sites. Rate of healing process was different in treated and untreated wounds and most striking difference was the appearance of appendages, sebaceous gland and hair follicle at some locations in HA-FS treated sites. Therefore, HA with fibrin can create an effective wound care matrix which promotes water retention and wound healing potential.

1. Introduction

Wound care products are in great demand for management of acute and chronic wounds and several such materials are under development. Primary function of wound dressing is to stop bleeding, absorb exudates, whereas additional bio-components such as adhesive proteins and growth factors can augment repair by means of their inherent wound healing properties. Examples of natural materials that are used for wound care include polypeptides, hydroxyl apatite, HA, fibronectin (FN), collagen, chitosan and alginates. Such materials have the advantage that they have low toxicity and low chronic inflammatory response.

Stimulation of the clotting cascade at the site of injury results in the proteolytic cleavage of fibrinogen by the enzyme thrombin, forming an insoluble fibrin clot that holds damaged tissues together and provides the provisional matrix for wound healing. In addition, the clot contains FN molecules that are present in plasma and bind to fibrin through fibrin-specific binding sites. Using an *in vitro* model, FN was found to be necessary for fibroblasts to express new integrin receptors and to migrate effectively on a collagen-fibrin matrix [1]. Growth factors and adhesive proteins enclosed within the provisional wound matrix help to stimulate fibroblasts to begin proliferation and synthesis of new collagen and other extra cellular matrix (ECM) components. Thus in acute wounds the provisional matrix plays several key roles, by providing a scaffold to direct cells into the injury and to stimulate them to proliferate, differentiate and synthesize ECM.

Glycosaminoglycans (GAGs) are major components of the ECM of the skin primarily composed of HA and dermatan sulphate with small amounts of chondroitin sulphate and heparan sulphate. The voluminous water of hydration associated with HA may be a mechanism for maintaining the normal hydration of the skin. The water attracting property of HA produces swelling pressure in ECM allowing the rapid diffusion of water-soluble molecules. Decreasing HA during aging imply a shrinkage accounting for dried and wrinkled appearance of aged skin [2]. Therefore, when HA is introduced to the wound bed in dry state, it may absorb the exudates from the injured tissue and retain the moisture to support various processes in wound healing which include proteolytic degradation of provisional matrix to increase cell migration and tissue remodeling.

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Earlier, we have established the use of lyophilized FS as a hemostat with wound healing properties for applications in liver stab injury, skin flap injury and also as a drug delivery vehicle [3,4]. An excess amount of thrombin is used for polymerization of fibrinogen which remains with the FS and thus acts as an excellent hemostat. As these sheets are lyophilized and stable at room temperature, it can be conveniently used for arresting blood loss. The FN which is inherently present in the cryoprecipitate used for producing fibrin clot is an added advantage of FS [5]. However, the FS prepared from the plasma-derived cryoprecipitate has no water sorbant component that can keep the wound bed moist. Therefore, degradation of fibrin and its absorption by surrounding tissue was delayed resulting in no specific advantage on wound healing process as compared to untreated controls.

The supportive role of exogenous HA in wound healing is attributed to its ability to promote epithelial migration, proliferation, regeneration and remodeling [6]. In fetal wounds, high level of HA persists until repair is completed. *In vivo* degradation of the HA in fetal wounds by hyaluronidase leads to fibrosis, inflammation, collagen deposition and angiogenesis [7]. Mast et al. also observed that HA regulates fetal fibroblast proliferation, and stimulated collagen and non-collagen protein synthesis [8]. Hyaluronan exists in free and tissue-bound form and there is a class of HA-binding proteins known as hyaladherens, among which HA-binding functions of fibrinogen, and collagen are well-known [9,10]. Various ECM components such as growth factors, adhesive proteins, and GAGs can be easily incorporated with fibrin clot through specific interactions of these molecules and FN. Fibronectin has binding sites for collagen, fibrin, heparin, HA and actin and is found in the ECM of many cells and is also a plasma protein (0.3 g/L) produced by the liver [11]. Its ability to bind to fibrin is mediated by transglutaminase activity and is extremely important in wound repair. Therefore, it is possible to use FN-containing exogenous fibrin clot as a delivery vehicle for HA to the injured tissue.

The objective of this study was to incorporate HA with fibrin sheet so that in addition to the hemostatic property, the sheets left on the wound bed may improve moisture retention which in turn can accelerate the healing process. We used rabbit-ear model for studying the progress of wound healing in presence of HA-FS and the possibility for therapeutic intervention for optimum healing with minimum scar. This report summarizes observations made during experimental evaluation of healing of acute dermal wounds in the presence of FS or HA-FS as compared to untreated ones.

2. Materials and methods

Fabrication of fibrin and HA-fibrin sheets: We made lyophilized sponge-like sheet of polymerized human fibrin. Polymerization of human fibrinogen (20 mg/ml) to fibrin was initiated by human thrombin (50 IU/ml). Cryoprecipitate that contain fibrinogen (20 ± 2 mg) and FN (200 ± 20 μg) per ml was prepared by freeze-thaw cycling and centrifugation [12], thrombin was prepared by ion exchange chromatography and both procedures were standardized in-house using screened human single donor unit plasma. Hyaluronic acid was purified in-house as per previously published method [13] from human umbilical cord and was characterized by FTIR (JASCO, Japan) spectroscopy. Hyaluronic acid film was made on a Zn–Se crystal and spectra were recorded between 4000 and 700 cm^{-1} in a FTIR spectrophotometer (JASCO, Model 6300, Japan, 0.07 cm^{-1} resolution) having a DLA-TGS detector. Purity was determined by HPLC analysis (Waters) using C18 column. Hyaluronic acid (5 μg/ml) was mixed with thrombin (50 IU/ml) and was added to an equal volume of fibrinogen (20 mg/ml) to form clot and the pre-formed clot in specific containers was freeze-dried to get sheets of required diameter.

Experimental wound and treatment: All animal experiments were conducted in New Zealand White rabbits with the permission from Institutional Animal Ethics Committee as per CPCSEA (Govt. of India) guidelines. Skin incisions (0.8 cm^2 area) were made using a template on dorsal aspect of ear (shown in Figs. 2 and 4). Three to four sites were created per ear and in the same animal one ear was used for controls and the other for treatment. Equal numbers of sites were used for treatment group and control. On treatment sites, FS or HA-FS was applied and control sites were left without any treatment. Animals were kept in individual cages with out any medication until euthanasia, which was done with excess intra venous dose of thiopentone.

In the case of FS treated sites three samples each were collected after 14 days, and 30 days of the experiment. For detailed histological analysis 12 explanted wound sites (from three animals) each were obtained from HA-FS treated sites and control making together 24 tissue samples (3 periods together). After gross photography, samples were fixed in 10% neutral buffered formaldehyde.

Histotechnology [14]: Tissues were processed in 70% alcohol (overnight), followed by three changes of acetone (20 min each),

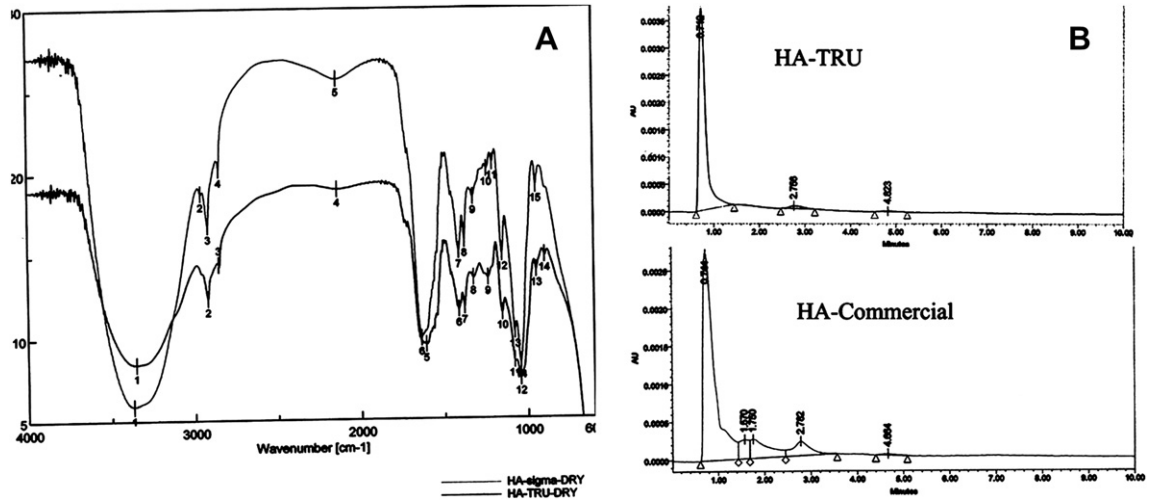

Fig. 1. Spectral & chromatographic features of hyaluronic acid. (A) IR spectrum of in-house purified HA and that from commercial source are overlaid for comparison. (B) elution pattern of in-house HA and commercial HA are shown for comparison.

two changes of xylene (10 and 15 min respectively) and two changes of paraffin wax (60 min each) before embedding in paraffin wax. Sections were then cut at a thickness of ~5 μm and stained with Harris's haematoxylin and eosin for routine histopathological screening of tissue by light microscopy. In addition, Masson's trichrome technique, Van Gieson's technique and Sirius Red staining were performed on histological sections for studying the nature of distribution of mature collagen.

Histopathology: the following tissue components or tissue reactions were identified in the wound site: (1) cartilage, (2) connective tissue between the cartilage and the original wound surface, (3) extent of re-epithelialization over the original wound surface, and (4) connective tissue between cartilage and the non-wound surface. Extent of formation of granulation tissue and neo-vasculature were graded. Deposition of collagen and re-epithelialization were also analyzed. The extent of inflammatory cell infiltration was noted. All parameters were graded as mild, moderate or severe (extensive) and conclusions were made.

Re-epithelialization: re-epithelialization was quantified in Haematoxylin and Eosin stained sections using Image-Pro software. The length of original wound and non-epithelialised wound length were measured and percentage of re-epithelialization was calculated by the formula (length of epithelialised wound/length of total wound) × 100. Same was repeated for each wound site and percentage of re-epithelialization was determined.

Neovasclarization: new blood vessels formed in the vicinity of the wound was quantified in Haematoxylin and Eosin stained sections using Image-Pro software by locating RBC area. For each wound site 12 high power fields were taken (high power objective, 40×) with all other microscope settings kept constant. The area with color intensity similar to RBCs within the newly formed blood vessels was quantified and percentage was calculated. This was repeated for more than 12 fields of wound tissue. The data obtained for each wound site was computed and analyzed using Student's *t* test to determine statistical significance.

Collagen distribution: collagen at the wound site was quantified morphometrically in Trichrome stained sections of wound tissue using Image-Pro software. Aniline-blue of Trichrome stains collagen 'blue' and this blue colored area was quantified for each wound site. More than 12 high power fields were taken (high power objective, 40×) for each sample and percentage of collagenous area was calculated and was analyzed using Student's *t*-test to determine statistical significance.

Confocal imaging: fluorescence of sections stained with Sirius Red and Van Geison's stains were analyzed. Specimens were excited with an Argon laser (488 or 514 nm) using a semi-automated laser scanning confocal microscope system (Zeiss LSM 510 META). Emitted fluorescence passing through 545 dichroic mirror and 610-long pass filter was detected with a single channel photomultiplier tube.

3. Results

All components of FS and HA were from human source isolated in-house. Purified HA showed spectral characteristic (Fig. 1A) which was comparable to the spectrum of HA that was procured from Sigma chemicals (USA). The purity of the product was further assured using HPLC analysis which showed a single strong elution peak, whereas there were prominent minor peaks in the case of commercial product that was used as reference (Fig. 1B), in which peak height of the major component was only ~60% of the purified product.

Rabbit ear was found to be an ideal site for making skin wounds, and creation of an injury resulted in significant bleeding as it is a highly vascular area. In the preliminary study the sites treated with FS was found to induce commendable haemostasis (Fig. 2A). The instant haemostasis was probably due to the excess thrombin in FS that clotted oozing blood and, with no sutures used, sheets

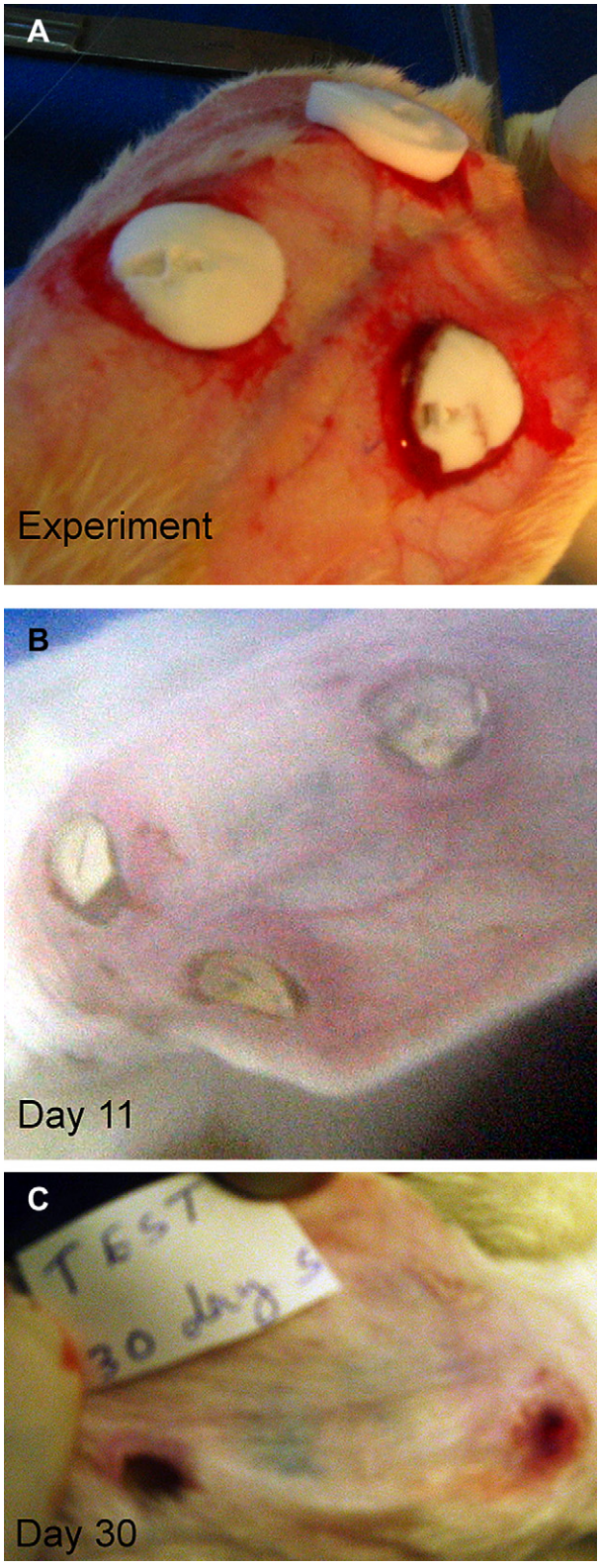

Fig. 2. Photographs of wounds during experiment period. (A) Fibrin sheet (FS) placed on wound bed inducing immediate clot formation. (B) FS treated sites after 11 days of experiment. (C) FS treated sites on 30th day of experiment.

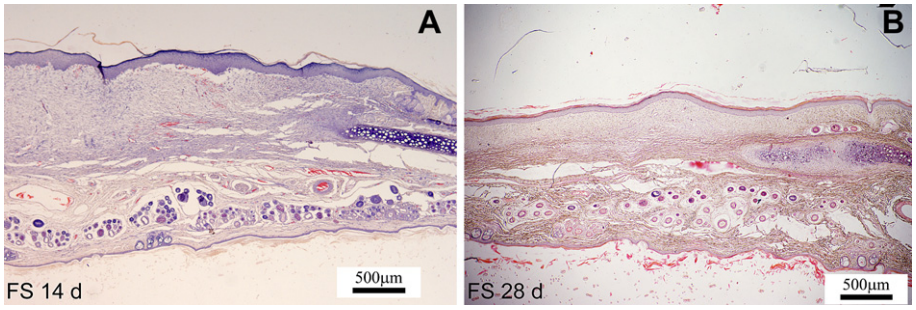

Fig. 3. Photomicrographs of FS treated sites. Representative histological sections of FS treated sites: (A) on day14 after the experiment; (B) on day 28 after experiment.

were secured at the wound bed by itself. It adhered adequately to the wound area and perfectly covered the wound bed because the size of the punch was matched with the size of the sheet and bleeding was arrested effectively. However, even after 10 days the FS were found to remain on the site of application without any significant degradation or desorption of fibrin (Fig. 2B), after 4 weeks the wound healing was not complete in FS treated sites (Fig. 2C). Extensive histopathological analysis of FS treated sites was not performed because the gross appearance was not encouraging; however, evaluation was done at two time-points (14 days & 28 days) by routine histological analysis (Fig. 3A & B). Basic healing was evident resulting in re-epithelialisation but with fibrosis at both time-points. The neo-epidermis was moderately thicker at 14 days (Fig. 3A) which resumed normality by the 28th day (Fig. 3B).

On the other hand, placed HA-FS stopped bleeding effectively (Fig. 4A), and gross appearance at all study intervals (Fig. 4B–D) showed marked difference compared FS treated sites and controls. When HA was mixed, FS was moist after 7days (Fig 4B) of experiment and by day 14 (Fig. 4C) remnants of fibrin was visibly less. Thick scab was seen on all control sites on day 14 which was reduced in size by day 28. Diameter of the wounded area was of similar size on 7 days in treated wound beds (0.78 ± 0.15 cm) and controls (0.80 ± 0.25 cm). But by day 14, size of the wound bed was significantly smaller in most of the treated sites, as compared to controls. The last period of the study was 4 weeks and by this time all the treated sites appeared near normal except a spot of 1–2 mm in the middle area. Controls were healed with visible scab and scarring (Fig. 4D) as compared to treated sites and difference between the groups was grossly appreciable by 4 weeks.

Detailed histopathological comparison was made between the HA-FS treated wounds and untreated control wounds, at 7 days (Fig. 5), 14 days (Fig. 6) and 28 days (Fig. 7) after the experimental surgery was performed. Sections indicated broken, non-continuous or inadvertently placed cartilage in some specimens, nevertheless there was progressive healing in all sections evaluated. Connective tissue healing was appreciable by the nature of granulation tissue, neo-vascularization and extent of fibrosis. Each of these parameters was graded as mild, moderate, severe and a quantitative estimate of the extent of the healing reaction was made. Special staining procedures were helpful for identification of connective tissue (Van Gieson's), mature collagen (Masson's trichrome) and all forms of collagen (Sirius Red).

Essentially, after seven days of the surgery, the healing was incomplete with plugs of exudates, moderate numbers of necrotic cells at the wound site and infiltration of lymphocytes and macrophages. Healing pattern was comparable in control sites and in test sites (Fig. 5A & B) which were plugged with healing scab but without continuous dermal epithelium. The infiltration of inflammatory cells was more frequent in the HA-FS treated sites than the control sites.

However, by 14th day (Fig. 6A & B), the re-epithelialization was complete and healing by fibrosis was evident. Desmoplastic reaction

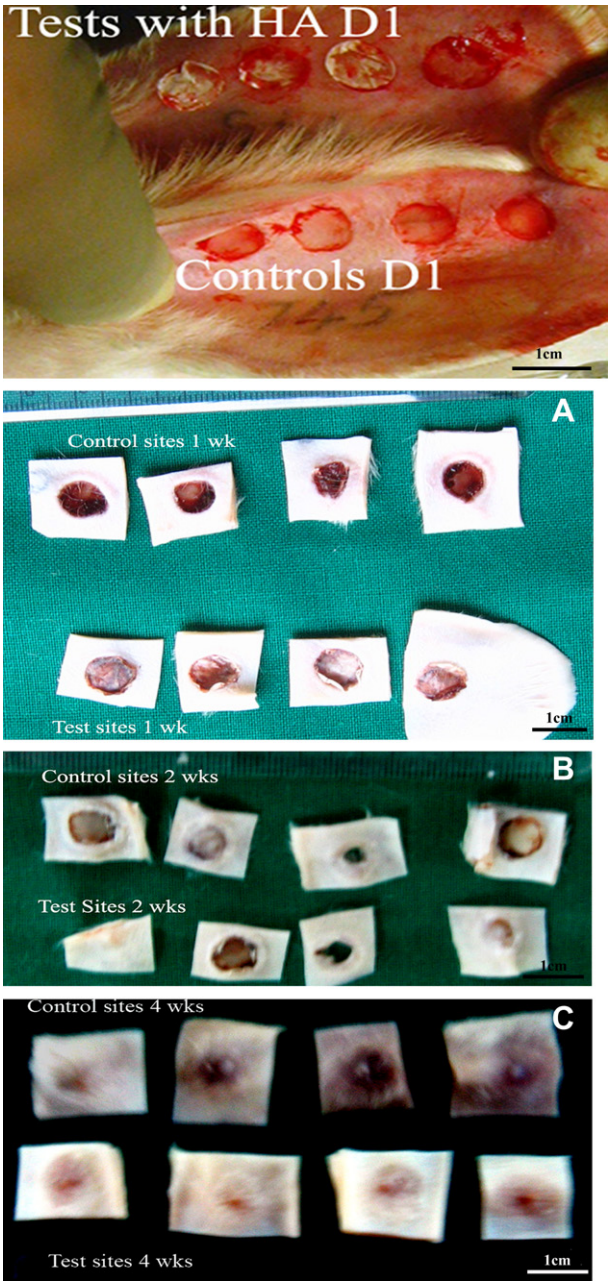

Fig. 4. Gross appearance of experimental sites after different intervals. (A) Test and control sites immediately after the experiment. Photographs of all the 24 explanted sites which consist of 12 controls and 12 tests are shown: (B) sites after 7d; (C) sites after 14d; and C, sites after 4wk.

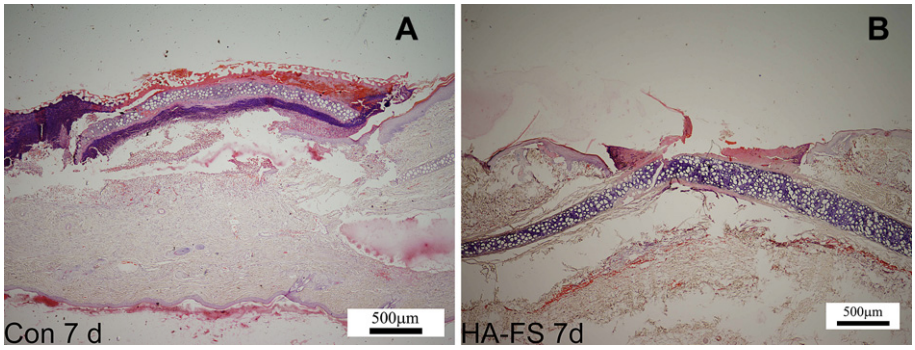

Fig. 5. Photomicrographs of healing reaction 7 days after surgery. (A) Harri's Haematoxiolin and eosin stained sections of: (A) test site and (B) control site (magnification is indicated in the photograph with scale bars).

with extensive collagen deposition was the principal feature at 14 days of wounding. In control sites, healing by fibrosis was prominant which indicates formation of scar tissue whereas excess granulation tissue with sprouting capillaries was consistently present in the sites treated with HA-FS. The nature of healing reaction appeared to be similar in all treatment groups. At 28 days (Fig. 7), there was remarkable difference in the healing pattern. The desmoplastic reaction was prominent in all samples and progressed with time. In addition, there was persistence of actively regenerating granulation in HA-FS compared to control. Complete healing by scarring was apparent in the control sites (Fig. 7A) but excessive granulation tissue with abundant neocapillaries was characteristically present in HA-FS treated sites (Fig. 7B) which demonstrate a possible attempt for resolution of newly laid collagen. The regeneration process was further supported by the development of appendages-like structure in the healing HA-FS treated tissue (Fig. 8A), which was not seen in any of the tissues or section from FS treated wound (Fig. 8B).

Histopathology sections were further analyzed quantitatively using Image Pro Plus™ software with images captured from various sections. Graphical representation of the data for extent of re-epithelialisation (Fig. 9), collagen deposition (Fig. 10) and neovascularisation (Fig. 11) corroborated the routine histomorphological observation and gave further quantitative support for the observations on the nature of the tissue reaction. At 7 day, re-epithelialization was more in treated wounds than that of controls (p value; 0.04). By day 14, re-epithelialization was nearly complete in treatment and control groups which progressed to intact epithelium by 28 day. This result indicates rapid re-epithelialization in HA-FS treated wounds (Fig. 9). Because the vascularization is measured based on the RBC area, it was more on day 7 and is similar in control and test. The RBC area showed reduction by day 14 in both cases, but by day 28 RBC area which corresponds to vascularization increased significantly in treated wounds as compared to control (Fig. 10). The data correlates well with histology pictures shown in Fig. 7 also. Presence of collagen was similar in control and treated sites on both 7 day and 14 day. But there was a significant difference between and HA-FS treated sites and control ($p \leq 0.05$) in collagen deposition by day 28 (Fig. 11).

Sections made from normal skin tissue sites of the same animals were used for comparison of collagen structures in remodelled wound area in a HA-FS treated site (Fig. 12). There was perceptible difference in the pattern and orientation of collagen in the healing tissue compared to the normal tissue. Two channel acquisition was done by artificial colouring of the field for studying the pattern and distribution of collagen (red) which showed wavy bundles arranged in compact fashion from a non-wounded area (Fig. 12A) while loose and branched mesh like pattern of neocollagen was seen in a wounded area (Fig. 12B). Collagen deposition was obvious in the HA-FS treated wound site. This imaging method was found to be suitable for identification of collagen deposition in conjunction with the progression of wound healing.

4. Discussion

The primary objective of this study was to evaluate effect of HA in fibrin sheet for its ability to improve water retention at the wound bed which in turn could promote degradation and bioresorption of fibrin. Dry wound bed could retard; cell migration, degradation of provisional matrix, deposition of new ECM and is not favourable for normal progression of wound healing. In the preliminary experiment, cryoprecipitate which consist of fibrinogen along with other coagulation proteins such as FN and FXIII

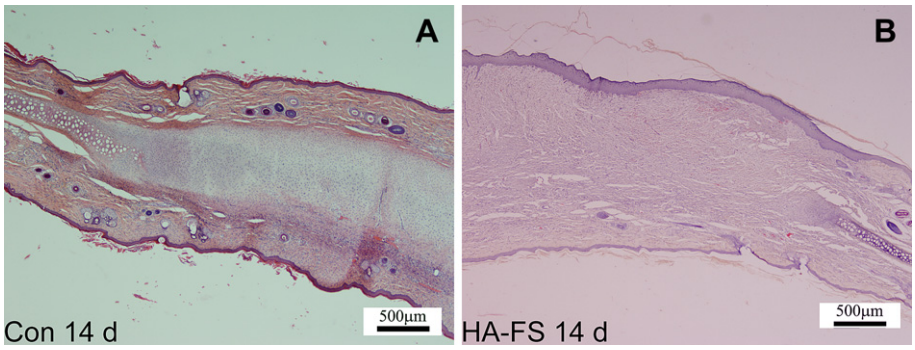

Fig. 6. Photomicrographs of healing reaction 14 days after surgery. A, Harri's Haematoxiolin and eosin stained sections of: A, test site and B, control site (magnification is indicated in the photograph with scale bars).

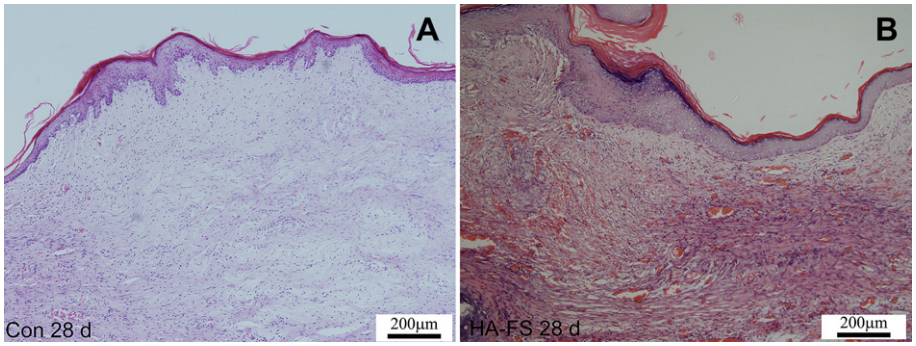

Fig. 7. Photomicrographs of healing reaction 28 days after surgery. (A) Harri's Haematoxiolin and eosin stained sections of: (A) test site and (B) control site (magnification is indicated in the photograph with scale bars).

was used for FS preparation and the preliminary results were not promising. Therefore, detailed analysis of wound healing was done with HA incorporated FS to compare treated wounds with control. When the sheet was modified with HA, the wound bed was visibly moist till the second week of application by which time the epithelialization was complete. Further, the histomorphometric evidences for vascularization, collagen deposition and hair follicle formation are presented to support the hypothesis that wound healing have improved when HA was incorporated with FS.

In a typical wound healing experiment, when the epithelium and the underlying connective tissue of the sub-cutis is removed, re-epithelialialization can be expected as early as 7 days depending on the size of the wound. Complete healing can happen between 14 and 28 days and the remodelling process with variable amounts of granulation tissue and neovascularisation of the connective tissue may take longer duration [15–17]. In the present study, re-epithelililisation was not complete by 7 days and incomplete epithelium indicated partial healing. Connective tissue healing was appreciated by the nature of granulation tissue, neovascularistaion and extent of fibrosis. Healing by fibrosis was the most noticeable difference between the wounds without exogenous fibrin (controls) against HA-FS treated sites.

After tissue injury, formation of a blood clot serves the dual role of restoring vascular integrity and serving as a temporary scaffold for the wound healing process. It has long been known that improper wound healing occurs in FXIII-deficient patients [18] and that fibroblast growth is impaired in clots formed from FXIII-deficient plasma [19]. Fibrin which is the main component of a blood clot is linked to the FN in the basement membrane through a glutamine residue and enables cells to migrate into clot during the repair process. So FXIII (transglutaminase) plays a role in cross linking of fibrin-fibrin and fibrin-FN. Fibronectin stimulates cell movement by mechanisms that are still not understood but which may be important to cell migratory events in embryonic development and wound healing [11]. In this study, use of stabilized exogenous fibrin sheet with FN which is inherently present in cryoprecipitate and the added HA together were found to be useful for promoting wound healing without fibrosis.

Since its discovery in human tissue, HA and its derivatives has been largely studied and applied in the biomedical arena. The appeal of this biopolymer has been accentuated by its high level of biocompatible nature. It has been used in viscosurgery to allow surgeons to safely create space between tissues. When incorporated into a neutral aqueous solution hydrogen bond formation occurs between water molecules and adjacent carboxyl and *N*-acetyl groups of HA. This imparts a conformational stiffness to the polymer, which limits its flexibility. The hydrogen bond formation results in the unique water-binding and retention capacity of the polymer.

The roles of HA in wound healing have been investigated intensively. Dollon et al. demonstrated both in vitro and in vivo models, to show that HA and FN are effective in enhancing fibroblast movement into a collagen sponge and in depositing collagen fibers during the early phases of wound healing [20,21]. Alternatively, HA might be part of a feedback loop that promotes cell proliferation and migration in actively growing tissues. Additionally, the role of HA in water homeostasis could favour tissue hydration, which has a positive effect on healing [20]. The percentage of HA in the skin is closely related to the hydration of skin. Hyaluronic acid retains water in the intercellular matrix of the dermis. It creates a visco-elastic solution that fills the spaces between the collagen fibers in the dermis [22,23]. Open wounds heal optimally in a moist, sterile environment. By keeping the

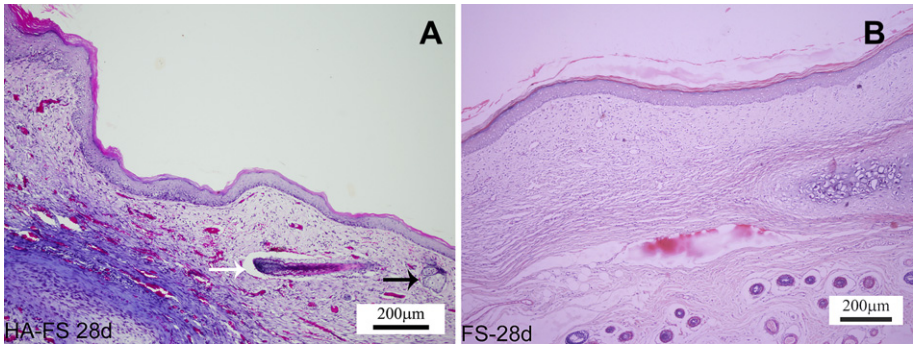

Fig. 8. Histomorphological details of healed HS-FA site. (A) Formation of tissue components on day 28 in HA-FS similar to hair follicle (white arrow) and sebaceous gland (black arrow); (B) section from FS site on day 28 (magnification is indicated in the photograph with scale bars).

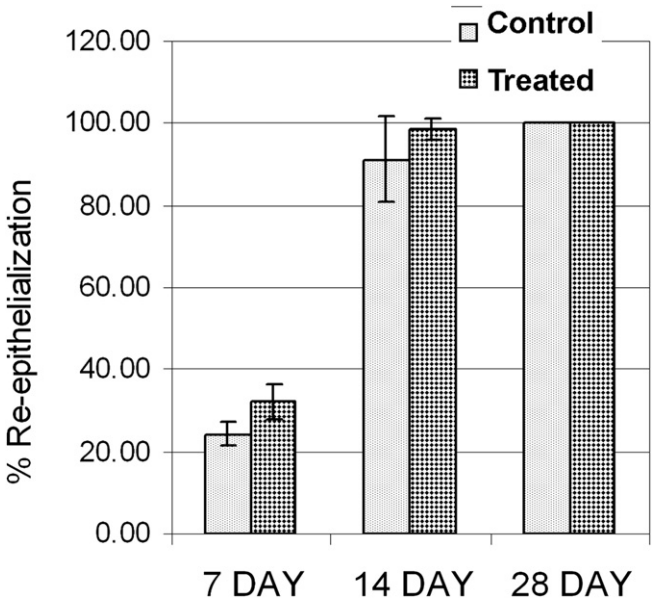

Fig. 9. Quantified data on re-epithelialisation. Data presented for each period is average ± S.D ($n = 12$). High resolution images were used for analysis.

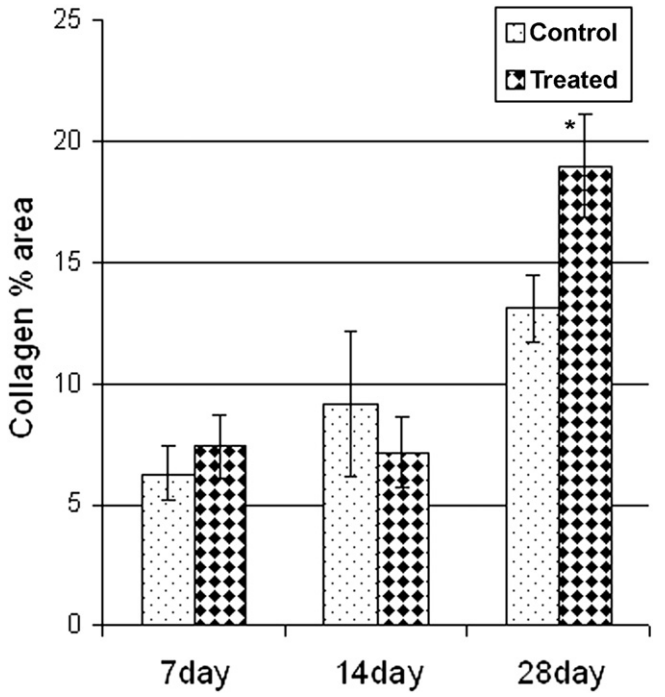

Fig. 11. Quantified data on collagen deposition. Data presented for each period is average ± S.D. ($n = 12$). High resolution images were used for analysis.

wound covered and moist without infection, desiccation necrosis and healing delay are prevented [24].

Some authors noted that exogenous HA may be beneficial in wound healing. The mechanism is unknown, but it has been hypothesized that HA promotes epithelial migration, as it seems to surround proliferating and migrating cells in regenerating, remodelling, or healing tissues [6,25]. Investigations that were carried out to understand effects of HA on wound healing indicated that both high molecular weight HA and its degradation products enhance cell migration and proliferation [26]. In this study we have taken advantage of the various binding sites on fibrin sheet for it to be used as a delivery system for HA. Fibrin and HA each have distinct properties that make them good candidates for use in tissue regeneration scaffolds. They probably have an additional synergistic effect on one another, thereby making the use of both materials in conjunction more desirable. Recently, Hayen et al. have determined that the presence of HA during fibrin polymerization changes the morphology of the resultant clot [27]. With HA present, a more porous and turbid clot was formed and it stimulated tumor cell migration. If the HA was added after fibrin clot formation, then there appeared to be no effect on cell motility [27]. Same group also determined that *in vitro* fibrin clot configuration can be correlated to the amount of capillary in-growth and endothelial cell migration within the clots [28]. Therefore, in this study addition of HA to the cryoprecipitate before it was clotted using thrombin seemed to produce an effective wound healing matrix. Both HA and fibrin promote angiogenesis through their degradation products rather than their polymerized forms, and degradation of the clot obviously requires a moist environment. Weigel et al. have determined that HA specifically binds fibrin(ogen) [29] which may play a role in changing the configuration of clots that have been formed in presence of HA [28]. Both fibrin and HA on their own have been shown to encourage cellular ingrowths into a wound site. However, we present here further evidence to demonstrate that by combining the two, additional benefits are seen such as formation of hair follicles and sebaceous glands. On the contrary such developments were not seen in our earlier report where fibrin clot was used for healing skin flap injuries [12]. We have not come across any reports which show hair follicle formation when any of the artificial or natural adjuncts are used for wound healing purpose.

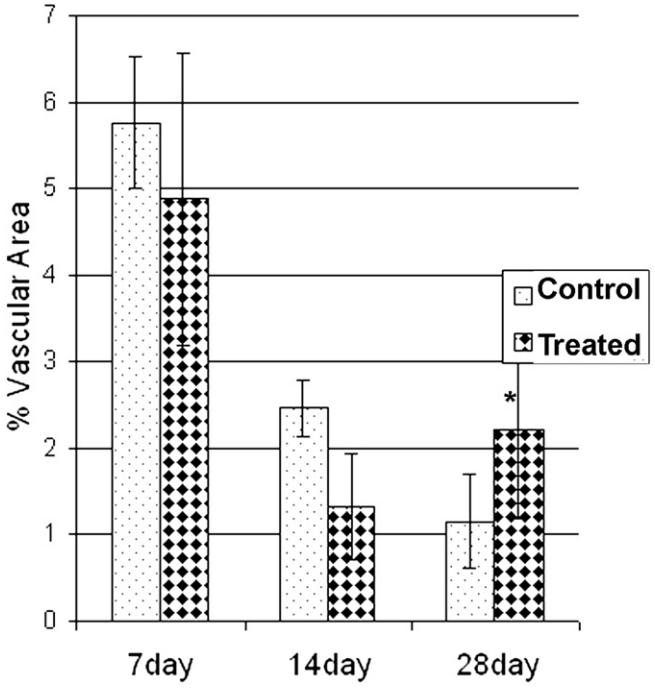

Fig. 10. Quantified data on neo-vascularization. Data presented for each period is average ± S.D ($n = 12$). High resolution images were used for analysis.

In conclusion, the experiment model described here is a good one for studying the cutaneous wound healing process. Laser induced fluorescence and confocal imaging is a potential tool for quantifying collagen in specific wound sites. A plethora of wound dressings are available for all types of wounds. No type has conclusively been shown earlier to accelerate healing more than the others. Maintenance of a moist and clean environment has been

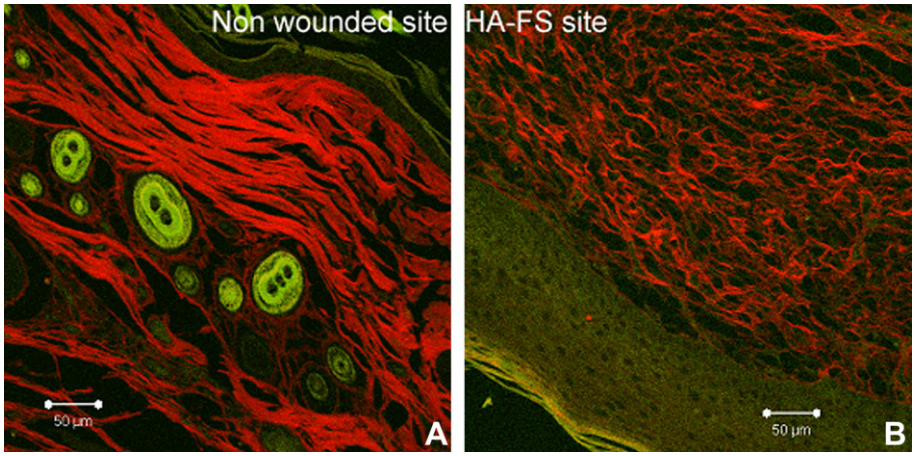

Fig. 12. Confocal images of collagen deposition. Tissues taken on 28th day of surgery was used for sectioning and imaging; red color is of collagen and all other components are colored green. (A) Section from non-wounded site; (B) section from wounded area. Sections were stained with Van Gieson's stain (laser-induced fluorescence images: excitation, 488 nm and emission, above 610 nm).

evidenced in this study. In normal rabbits even without the treatment, wound healing may take place in the natural course but this model may have more application in the case of diabetic or burn wounds which are difficult to heal.

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